LFA-1 Contributes to Signal I of T-Cell Activation and to the Production of T_h1 Cytokines

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The β_2 integrins are important for both transendothelial migration of leukocytes and T-cell activation during antigen presentation. In T cells, triggering of leukocyte functional antigen-1 (LFA-1) is required for full activation and T-helper (Th)1/Th2 differentiation. We used CD18-deficient ($CD18^{-/-}$) mice to examine the role of LFA-1 in the activation of T cells. Compared with wild-type controls, $CD18^{-/-}$ T cells proliferated normally when stimulated with antibodies against CD3 and CD28, but secreted significantly less IFN-γ and IL-2 than their wild-type counterparts. However, when T cells were stimulated with dendritic cells (DCs) that provide additional LFA-1 ligation, the proliferation of $CD18^{-/-}$ T cells was significantly reduced, whereas cytokine production remained impaired. The diminished proliferative capacity of $CD18^{-/-}$ T cells could be fully compensated for by additional triggering of the T-cell receptor, but not by additional stimulation through the costimulatory molecule, CD28. Thus, ligation of LFA-1 on T cells participates in regulation of Th1 cytokines *in vivo*. In addition, LFA-1 primarily exerts an effect as an enhancer of TCR signalling and does not facilitate classical costimulation.

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INTRODUCTION

β2 integrins (CD11/CD18) are heterodimeric leukocyte adhesion molecules that are exclusively expressed on hematopoietic cells and responsible for cell–cell contacts between leukocytes as well as for contacts between leukocytes and endothelial cells (Anderson *et al.*, 1986; Hynes, 1992). The common β-chain (CD18) associates with four different α-subunits, α-L, α-M, α-X, and α-D, forming distinct functional heterodimers termed leukocyte functional antigen-1 (LFA-1; CD11a/CD18), Mac-1 (CD11b/CD18), gp150/95 (CD11c/CD18), or CD11d/CD18 (Arnaout, 1990; Hynes, 1992; Van der Vieren *et al.*, 1995). These interact with more than 20 ligands, of which the most prominent belong to the ICAMs (Carlos and Harlan, 1994; Springer, 1994). LFA-1 is the only β2 integrin expressed on T cells.

An adhesion between T cells and antigen-presenting cells (APCs) is necessary for the formation of the immunological synapse and is mediated by T-cell surface molecules, including LFA-1 and ICAM-1, -2, and -3 (in humans) (Vazeux *et al.*, 1992; Springer, 1995). In contrast to the extensive data available concerning signals involved in TCR and CD28 ligation (Linsley and Ledbetter, 1993; Acuto *et al.*, 2003), much less is known about specific signals generated by adhesion-mediated events involved in the contact of T cells with APCs. Optimal proliferation of T cells and enhancement of IL-2 production requires adhesive interactions between costimulatory receptors on T cells and their counter-ligands on APCs. In addition to TCR and CD28 stimulation, the model systems that are used to study T-cell activation have shown that increasing antigen density by more than 10,000-fold does not initiate naïve $CD4^+$ T-cell proliferation or cytokine synthesis in the absence of ICAM–LFA-1 interaction (Abraham *et al.*, 1999). In addition, Kandula and Abraham (2004) showed that LFA-1 was required on $CD4^+$ T cells for optimal activation. However, the specific functional consequences and mechanisms of an LFA-1-mediated ''costimulatory'' signal are not well defined. ICAM–LFA-1 interactions have also been implicated to regulate T-helper cell differentiation (Camacho *et al.*, 2001; Chirathaworn *et al.*, 2002; Smits *et al.*, 2002; Perez *et al.*, 2003) predominantly supporting T_h1 induction, thereby suppressing T_h2 differentiation of T cells (Maraskovsky *et al.*, 1992; Semnani *et al.*, 1994; Salomon and Bluestone, 1998; Luksch *et al.*, 1999; Jenks and Miller, 2000). We recently showed that lack of the β2 integrin subunit also markedly impairs T-cell extravasation in mice with a CD18-null mutation ($CD18^{-/-}$; Grabbe *et al.*, 2002). Even when housed under pathogen-free conditions, mice develop lymphadenopathy and splenomegaly with increasing age, accompanied by activated peripheral T cells (Grabbe *et al.*, 2002).

In this study we now show that $CD18^{-/-}$ T-cell activation is profoundly impaired when stimulated with dendritic cells

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(DCs) that provide LFA-1 ligation in wild-type (wt) T cells, but not when stimulated with anti-CD3 and anti-CD28. In addition, IFN-γ and IL-2 production is significantly diminished in the absence of CD18 and in the presence of DCs. This impairment can be compensated for by increasing signal I (TCR) of T-cell activation, but not by increasing signal II (''classical co-stimulation''). Hence, LFA-1 signal contributes to signal 1 of T-cell activation and is required for both full T-cell proliferation and for T_h1 differentiation.

RESULTS

LFA-1 is required for T-cell adhesion to DCs

We co-cultured $CD18^{-/-}$ and $CD18^{+/+}$ T cells with syngeneic DCs from $CD18^{+/+}$ mice, which besides TCR ligation also provide LFA-1 ligation through ICAMs that are expressed on DCs. We first analyzed the physicodynamics of T-cell–DC interactions in a three-dimensional (3D) collagen gel matrix. We seeded either $CD18^{+/+}$ or $CD18^{-/-}$ T cells together with DCs into collagen gels and analyzed T–DC contact times as described earlier (Gunzer *et al.*, 2000). Contact times of $CD18^{-/-}$ T cells with DCs were significantly diminished when compared with $CD18^{+/+}$ T cells (Figure 1a). In a separate approach, we analyzed T–DC clusters in liquid culture (Figure 1b). In addition, in this approach $CD18^{-/-}$ T cells formed smaller clusters with DCs when compared with $CD18^{+/+}$ T cells. Thus, lack of LFA-1 on T cells led to shorter T–DC contact duration and to less adhesiveness. This was independent of the degree of DC maturity, as we obtained comparable results with immature DCs (data not shown).

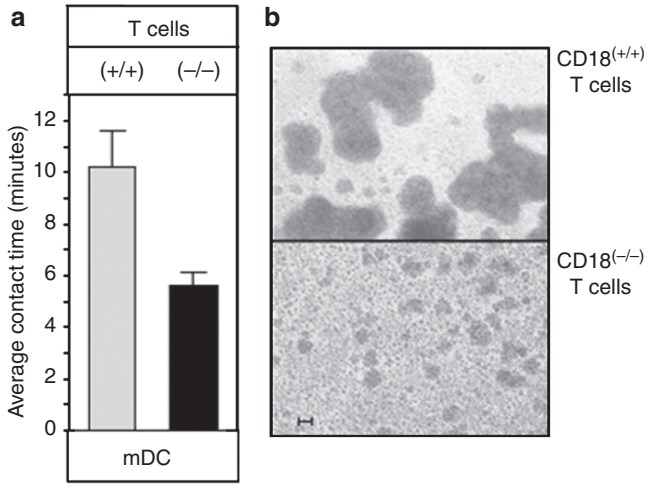

Figure 1. Impaired binding activity of $CD18^{-/-}$ T cells. (**a**) Three-dimensional collagen gel co-cultures containing 4×10^4 T cells and 4×10^4 syngeneic mature dendritic cells (DCs) from $CD18^{+/+}$ mice were monitored for 72 hours using time-lapse videomicroscopy and analyzed for the duration of randomly selected individual physical interactions of T cells and DCs. Contact times of 50 individual T–DC pairs were analyzed and expressed as mean value (± SEM). (**b**) For homotypic aggregation, 2×10^6 syngeneic DCs ($CD18^{+/+}$) and either 10×10^6 $CD18^{+/+}$ or $CD18^{-/-}$ T cells were plated on six-well plates for 24 hours and photographed directly from the culture plates (scale bar = 0.1 mm). Photographs are representative of five independent experiments.

Lack of LFA-1 leads to impaired signal 1 in DC-induced T-cell activation

We wondered whether the diminished and shortened adhesiveness of CD18-deficient T cells would also translate into impaired T-cell proliferation. T cells from either $CD18^{-/-}$ or $CD18^{+/+}$ mice were co-cultured with allogeneic DC and proliferation was measured. $CD18^{-/-}$ T cells proliferated significantly less (Figure 2a and b) than $CD18^{+/+}$ T cells, when co-cultured with DC. This could be due to insufficient TCR-mediated T-cell activation at the DC–T-cell interface (''signal 1'') or due to a lack of costimulation (''signal 2''). To test these possibilities, we added either anti-CD3 antibody, anti-CD28 antibody, or both antibodies (Figure 2c) to the T–DC co-cultures, because exogenous addition of antibodies can maximize the respective signal even in the absence of LFA-1. Interestingly, adding anti-CD3 antibody to the co-culture completely compensated for the deficiency in proliferation of $CD18^{-/-}$ T cells, whereas additional stimulation through CD28 alone did not compensate at all for the reduced ability of $CD18^{-/-}$ T cells to proliferate (Figure 2a and c). Triggering of both pathways did not further increase proliferation of $CD18^{-/-}$ T cells (Figure 2a and c). To exclude the possibility that this effect is due to the activation status of DCs, we also used immature DCs, yielding the same results as with mature DCs (data not shown). Annexin V and propidium iodide staining of the co-cultures showed (Figure 2d) no difference in apoptotic cells in the co-cultures, either with $CD18^{+/+}$ T cells or with $CD18^{-/-}$ T cells, respectively.

ICAM-1 on DC is essential for full LFA-1-dependent T-cell activation

We next blocked ICAM-1 in our co-cultures of BALB/c DCs with either $CD18^{+/+}$ or $CD18^{-/-}$ T cells to verify the importance of ICAM-1–LFA-1 interaction for T-cell activation (Figure 3). When ICAM-1 antibody was used in the culture with $CD18^{+/+}$ T cells and DCs, the proliferation that was induced by DCs was almost completely abrogated (Figure 3a, upper dot plots, and Figure 3b), suggesting that the ICAM-1–LFA-1 interaction is essential for full T-cell activation. However, little $CD18^{-/-}$ T-cell proliferation was detectable even without ICAM-1–LFA-1 binding in some experiments (Figure 3a lower dot plots, and Figure 3b).

Lack of LFA-1 leads to impaired T_h1 differentiation

As T-cell proliferation was impaired significantly (Figure 2a and b) but cells did not die of apoptosis (Figure 2d), we asked whether T-cell differentiation was also changed and determined their cytokine production. Production of the T_h1 cytokines IFN-γ and IL-2 was significantly reduced in $CD18^{-/-}$ T cells when stimulated with allogeneic DCs (Figure 4). IFN-γ and Il-2 production were in part but not fully dependent on LFA-1–ICAM-1 interaction as determined from experiments using anti-ICAM-1 antibody. We also detected reduced IL-4 (a T_h2 cytokine) production in $CD18^{-/-}$ T cells, although the differences between $CD18^{+/+}$ and $CD18^{-/-}$ T cells did not reach statistical significance. Thus, ICAM-1–LFA-1 interaction promotes T_h1 differentiation but has no direct effect on T_h2 development.

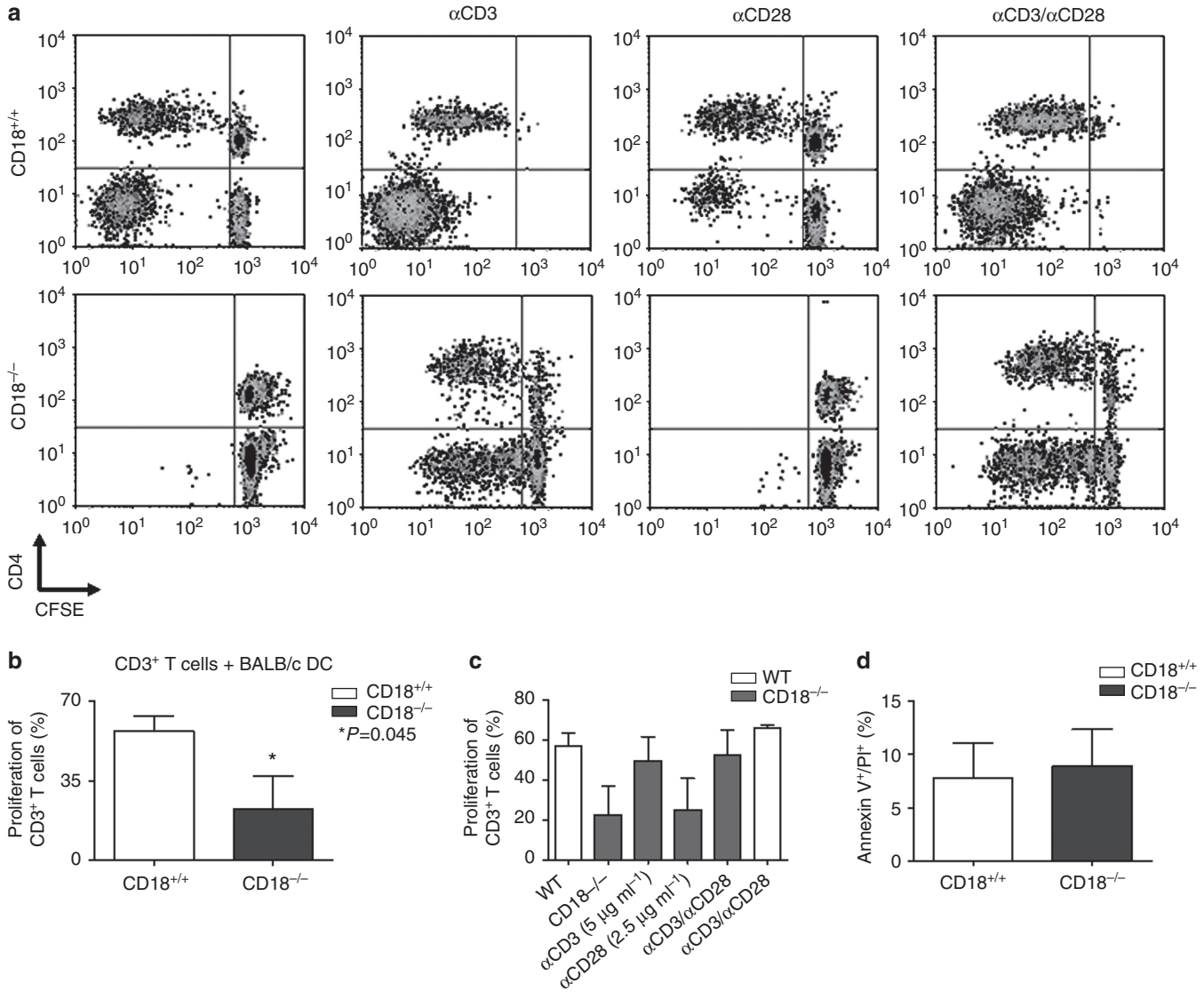

Figure 2. Leukocyte functional antigen-1 (LFA-1) contributes to "signal 1" of T-cell activation. (**a**) 1×10^5 Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled splenic $CD3^+$ T cells were co-cultured with allogenic dendritic cells (DCs; BALB/c) in a ratio of 40:1 (T:DC) for 5 days. Where indicated, cultures were additionally stimulated with anti-CD3 (5 μg ml^{-1}) and/or anti-CD28 (2.5 μg ml^{-1}) antibodies. Cells were stained for CD4, and proliferation (CFSE dilution) was assessed using FACS. Data show one representative example of three independent experiments. (**b**) Proliferation (CFSE dilution) of $CD3^+$ T cells from either $CD18^{+/+}$ or $CD18^{-/-}$ mice after 5 days co-culture with allogeneic BALB/c DC (T and DC in a ratio of 40:1). Graph shows mean and SEM ($n = 9$) of three independent experiments. (**c**) As in **b**, but additionally cultured with α-CD3 and α-CD28 antibodies. Mean and SEM ($n = 9$) of three independent experiments is shown. (**d**) Co-cultures from **b** were stained with Annexin V and propidium iodide (PI) and analyzed for apoptotic cells using FACS. The percentage of Annexin V^+/PI^+ cells after 5 days of co-culture is shown. Graph represents mean and SEM of three independent experiments.

DC-independent T-cell activation is not impaired in $CD18^{-/-}$ T cells

ICAM-1 is also expressed on T cells, and we were interested to examine how important T-cell-expressed ICAM-1 is for T-cell proliferation. Therefore, we stimulated T cells with antibodies against CD3 and CD28, but in the absence of DCs, and assessed T-cell proliferation (Figure 5a). Where indicated, plate-bound recombinant ICAM-1-Fc protein was used to stimulate T cells additionally (Figure 5b). When stimulated with anti-CD3 and anti-CD28 antibodies, but without DCs, proliferation of either $CD18^{+/+}$ or $CD18^{-/-}$ T cells did not differ (Figure 5a and b), suggesting that CD18-deficient T cells do not have an inherent defect in proliferative capacity or are genuinely impaired in TCR-mediated signal transduction. In addition, additional stimulation with ICAM-1-Fc did not further enhance T-cell proliferation, at least at the stimulatory conditions used in this study, suggesting that LFA-1–ICAM-1 interaction may be essential in the context of the immunological synapse but not sufficient for optimal T-cell activation.

Although T-cell proliferation was normal in $CD18^{-/-}$ T cells that were stimulated in the absence of a DC surface, merely by anti-CD3/anti-CD28, cytokine production still was impaired, as shown for IFN-γ, IL-2, and IL-4 in Figure 6.

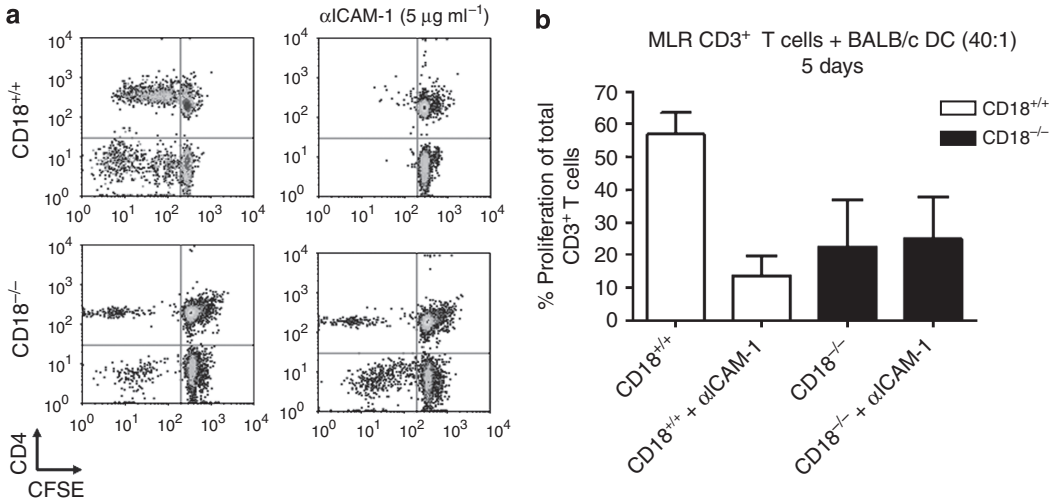

Figure 3. Leukocyte functional antigen-1 (LFA-1) and ICAM-1 interaction is essential for dendritic cell (DC)-induced T-cell proliferation. (**a**) Splenic $CD3^+$ T cells from either $CD18^{+/+}$ (C57BL/6) or $CD18^{-/-}$ mice were prepared and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) as described in Material and Methods. 1×10^5 T cells were then co-cultured with allogeneic DC (BALB/c) in a ratio of 40:1 (T:DC) for 5 days. Where indicated, 5 μg ml^{-1} α-ICAM-1 antibody was added additionally. The cells were then stained for CD4, and proliferation (CFSE dilution) was assessed using FACS. Data show one representative example of three independent experiments. (**b**) Quantification of **a**. Graph shows mean and SEM ($n = 9$) of three independent experiments.

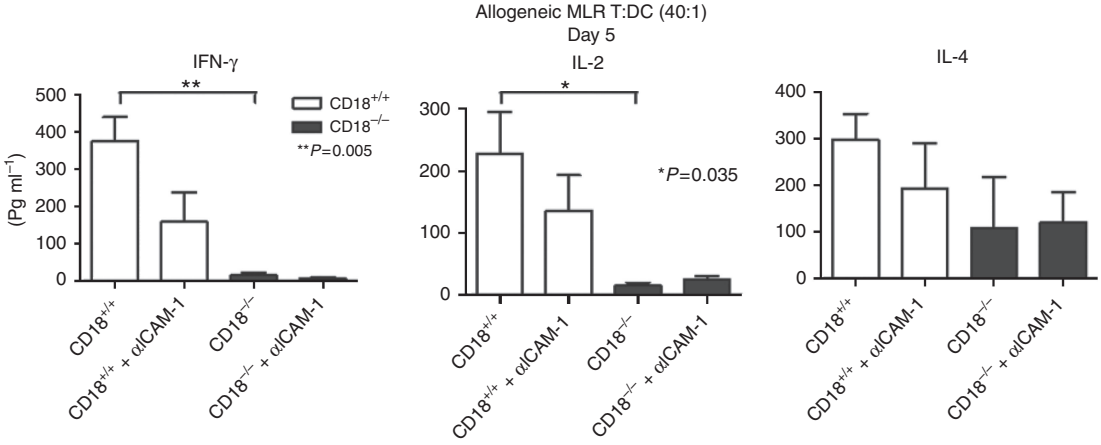

Figure 4. Cytokine production of $CD18^{-/-}$ T cells induced by allogeneic BALB/c dendritic cells (DCs). 1×10^5 Quiescent T cells were incubated with allogeneic DC (BALB/c) in a ratio of 40:1 (T:DC) for 5 days. Where indicated, 5 μg ml^{-1} α-ICAM-1 antibody was additionally added. On day 5, supernatants were analyzed for the content of IFN-γ, IL-2, and IL-4 using the cytometric bead array technology. Data are expressed as mean and SEM ($n = 9$) of three independent experiments.

Thus, the T_h1 differentiation defect of $CD18^{-/-}$ T cells is not fully dependent upon DC contact.

DISCUSSION

Relevance of β_2 integrins for T-helper cell polarization

In this study, we examined the role of LFA-1 for priming and differentiation of naïve T cells. In part, this issue has also been addressed by other researchers before, although with partly divergent results. Experiments using specific mAb to block the β_2 integrin LFA-1 *in vivo* suggest that signalling through LFA-1 has an important role in polarization toward type 1 immune responses (Maraskovsky *et al.*, 1992; Semnani *et al.*, 1994; Salomon and Bluestone, 1998; Jenks and Miller, 2000; Smits *et al.*, 2002). In contrast, LFA-1-deficient mice seem to be biased toward T_h1 development in a model of *Listeria monocytogenes* infection, which seems to be mediated by elevated IL-12 production by granulocytes (Emoto *et al.*, 2003). Similarly, CD18-deficient mice similarly seem to be biased towards T_h1 development in a model of experimental leishmaniasis by elevated IL-12 release from CD18-deficient macrophages (Schonlau *et al.*, 2000). Lee *et al.* (2003) showed that in $CD18^{-/-}$ mice, both T_h1 and T_h2 cells can be primed, and that migration of T_h2 cells to sites of inflammation, but not of T_h1 cells, is impaired.

In this study, we examined whether $CD18^{-/-}$ T cells were already committed to a certain T-helper type *in vivo*, and

whether they have similar functions when compared with wt T cells. When stimulated independently of LFA-1 engagement by DCs, proliferation of quiescent T cells from CD18$^{-/-}$ mice was equal to that of wt T cells (Figure 5). This result is consistent with the study of Lee *et al.* (2003) who also determined equal proliferation capacity when T cells were stimulated in the absence of LFA-1 engagement. Schonlau *et al.* (2000) also showed that T cells from *L. major*-infected CD18$^{-/-}$ mice regain their proliferative capacity in the absence of CD18, but only after addition of IL-2. Thus, under conditions in which the topographical redistribution of membrane molecules is irrelevant for TCR triggering, β_2 integrins are not required for T-cell activation. In contrast, the cytokine profiles differed greatly between CD18$^{-/-}$ and wt T cells. In the absence of triggering by DCs, wt T cells produced more IFN-γ and IL-2 than CD18$^{-/-}$ T cells. In addition, production of IL-4 was also reduced in CD18$^{-/-}$ T cells, although not as prominent (Figure 6). The diminished secretion of IFN-γ by CD18$^{-/-}$ T cells was not due to impaired capacity to produce this cytokine, as IFN-γ-producing cells were not diminished when stimulated with phorbol 12-myristate 13-acetate/ionomycin (data not shown). This result is consistent with Schonlau *et al.* (2000) who found an equal increase in IFN-γ production from wt and CD18$^{-/-}$ T cells after stimulation with IL-2 (by-passing the need for membrane interactions). The difference in IFN-γ production that is not observed by Lee *et al.* (2003) might be due to the use of allogeneic DCs in our study. When stimulated with anti-CD3/anti-CD28, the differences in IFN-γ production become similar to what was determined by Lee *et al.* (2003).

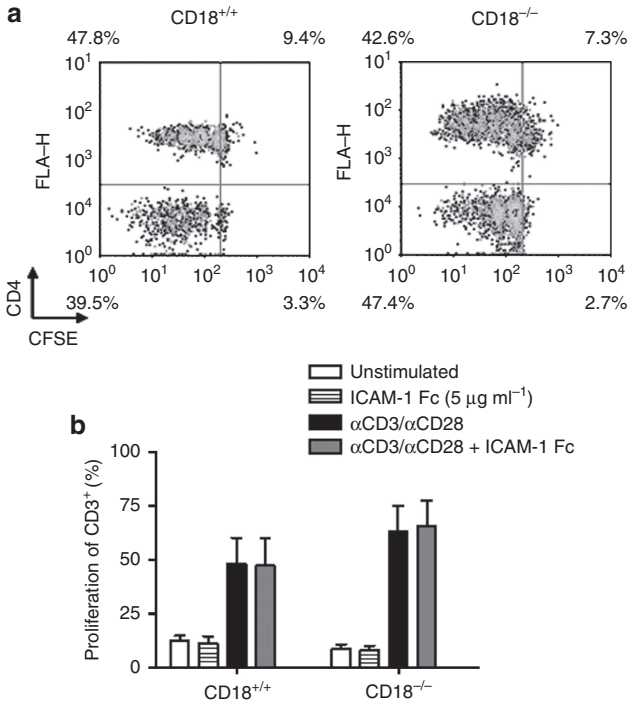

Figure 5. α-CD3/α-CD28-induced proliferation of CD18$^{-/-}$ T cells. (**a**) Splenic CD3$^+$ T cells were prepared and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) as described in Materials and Methods. 1×10^5 T cells were then cultured with anti-CD3 (5 μg ml^{-1}) and anti-CD28 (2.5 μg ml^{-1}) antibodies, respectively. After 7 days of culture, the cells were harvested. Cells were stained for CD4, and proliferation (CFSE dilution) was assessed using FACS. Data show one representative example of four independent experiments. (**b**) Splenic CD3$^+$ T cells were incubated as in **a** or left untreated. Where indicated, cells were additionally stimulated with plate-bound recombinant ICAM-1-Fc chimera protein (10 μg ml^{-1}) to bind leukocyte functional antigen-1 (LFA-1). After 7 days of culture, the cells were analyzed for proliferation (CFSE dilution) using FACS. Graph contains mean and SEM ($n = 7$) of four independent experiments.

Relevance of β_2 integrins for TCR signalling and costimulation

The adhesive function of LFA-1 to bind to its counter receptors, the ICAMs, has been studied very intensively (Hogg *et al.*, 2002; Porter *et al.*, 2002). When we sought to analyze LFA-1-dependent functions of T cells, we used DCs to provide additional ICAM–LFA-1 ligation on the surface of T cells. As expected, in our 3D collagen gel matrix, the contact times of CD18$^{-/-}$ T cells with DCs were lower compared with wt T cells. Similarly, clustering of T cells with DCs in liquid culture was also impaired, emphasizing the relevance of LFA-1 as a cell–cell adhesion molecule. In addition to this function, more recent studies have suggested an active role of LFA-1 in intracellular signalling, leading to T-cell activation (Zuckerman *et al.*, 1998; Ni *et al.*, 1999; Ragazzo *et al.*, 2001; Perez *et al.*, 2003). To further analyze this feature, we performed mixed lymphocyte reactions with allogeneic DCs. Proliferation of CD18$^{-/-}$ T cells was

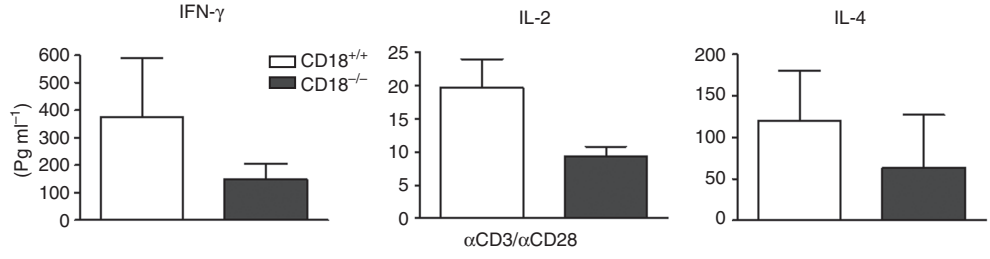

Figure 6. Cytokine production of CD18$^{-/-}$ T cells induced by α-CD3/α-CD28. 1×10^5 Quiescent T cells were incubated with α-CD3 (5 μg ml^{-1}) and α-CD28 (2.5 μg ml^{-1}) antibodies for 7 days. Supernatants were taken and analyzed for the content of IFN-γ (wild type (wt): 375.9 ± 213.9; CD18$^{-/-}$: 148.9 ± 56.9, $P = 0.36$), IL-2 (wt: 19.7 ± 4.3; CD18$^{-/-}$: 9.3 ± 1.5, $P = 0.09$), and IL-4 (wt: 120 ± 60.6; CD18$^{-/-}$: 63.4 ± 45.3) using cytometric bead array technology (FlexSets). Data are expressed as mean and SEM ($n = 9$) of three independent experiments.

significantly reduced when compared with wt T cells and this effect was not due to increased apoptosis in $CD18^{-/-}$ T cells. Thus, LFA-1 triggering on wt T cells amplifies T-cell proliferation in response to physiological stimulation of the TCR (signal I) and CD28 (signal II) by DCs. This was in clear contrast to TCR- and CD28-induced T-cell proliferation in the absence of DCs, which was not different between $CD18^{-/-}$ and wt T cells (Figure 5a and b). Thus, LFA-1 ligation contributes to the physiological triggering of the TCR and/or costimulatory receptors, but not to pharmacological T-cell stimulation with α-CD3 and α-CD28, at least when saturating concentrations of anti-CD3 and anti-CD28 were used. This suggests that LFA-1 is required for reorganization of TCR molecules at the plasma membrane, which is necessary for efficient T-cell activation. In accordance with this interpretation, LFA-1 has been shown to be one key molecule within the immunological synapse (reviewed in Sims and Dustin, 2002). As it is still unclear whether TCR-mediated and costimulatory signals are distinct from each other and whether both are transmitted through synapse formation, we analyzed whether the diminished proliferative capacity of $CD18^{-/-}$ T cells was due to reduced signal I and/or signal II of T-cell activation. To amplify signal I, we added anti-CD3 antibody and to amplify signal II, we added anti-CD28 antibody to the mixed lymphocyte reaction, respectively. Interestingly, enhancement of signal I fully compensated for the lack in proliferative capacity of LFA-1-deficient T cells, whereas augmentation of signal II completely failed to do so. Thus, LFA-1 primarily exerts an effect as an enhancer of TCR signalling, suggesting that LFA-1 signalling lowers the threshold of T-cell activation by enhancing TCR-induced signalling, whereas it does not facilitate costimulation through CD28. It may do so by providing the adhesive properties required for the formation of the immunological synapse. Indeed, impaired synapse formation may also contribute to the T_h1, but not T_h2, defect in $CD18^{-/-}$ mice. As T_h2 cells are not as efficient as T_h1 cells in generating the immunological synapse, the T_h2 response may not be as dependent on the immunological synapse as the T_h1 response. This would also be consistent with the studies of Perez *et al.* (2003) who showed the contribution of LFA-1 signalling to intracellular pathways that can also be triggered by the TCR. LFA-1, on the other hand, does not activate, e.g., the p38 mitogen-activated protein kinase pathway (Perez *et al.*, 2003) that is one of the targets of CD28 costimulation (Zhang *et al.*, 1999).

In aggregate, our results indicate diverse roles for LFA-1 in T-cell activation and differentiation. First, T_h1-cytokine production by T cells is inherently regulated by LFA-1 signalling. Second, LFA-1 not only facilitates cell–cell contact, but also actively enhances TCR signalling. Third, proliferation and cytokine production of T cells are both influenced by LFA-1 signalling, but are regulated independent of each other.

MATERIALS AND METHODS

Mice

$CD18^{-/-}$ and $CD18^{+/+}$ (wt) control mice of C57BL/6 ($H\text{-}2^b$) origin were used at the age of weeks 16–20 as described (Scharffetter-Kochanek *et al.*, 1998). BALB/c mice were used for bone marrow-derived DC preparation. All animal studies were approved according to the federal regulations (no. A39/2000 Bezirksregierung Münster).

Antibodies and reagents

All antibodies were purchased from BD Pharmingen (Heidelberg, Germany), including anti-CD11c-APC (HL-3), anti-CD3-PerCP (145-2C11), anti-CD4-APC (L3T4), anti-CD28 (35.51), anti-CD80-FITC (16-10A1), anti-CD86-PE (GL1), anti-ICAM-1 (3E2), Annexin V-FITC, and cytometric bead array for cytokine detection (FlexSet: IFN-γ, IL-2, and IL-4). For cell separation, pan-T-cell isolation kit ($CD3^+$) was from Miltenyi Biotech (Bergisch-Gladbach, Germany). Bovine type I-collagen (Vitrogen 100) was purchased from Cohesion Technologies (Palo Alto, CA, USA). Carboxyfluorescein diacetate succinimidyl ester was from Invitrogen (Karlsruhe, Germany). Propidium iodide was purchased from Sigma (Taufkirchen, Germany).

Generation of DCs and isolation of T cells

DCs were generated essentially as described (Inaba *et al.*, 1992; Labeur *et al.*, 1999). To generate mature DCs, bone marrow cells were cultured for 6 days in the presence of 150 U ml^{-1} GM-CSF and IL-4 (both from conditioned cell culture supernatants kindly provided by Thomas Blankenstein, MDC Berlin, Germany), with subsequent exposure to lipopolysaccharide (100 ng ml^{-1}) for 48 hours, as described (Labeur *et al.*, 1999). All DC preparations used were >80% pure with respect to CD11c expression (data not shown).

Spleens and lymph nodes of $CD18^{+/+}$ or $CD18^{-/-}$ mice were taken and T cells were prepared as described (Gunzer *et al.*, 2001). For isolation of T cells from spleen, pan-T-cell isolation kit ($CD3^+$ T-cells, Miltenyi Biotech) was used according to the manufacturer's instructions and cells were separated using Magnetic Cell Separation (MACS) technology (Miltenyi Biotech). Cells were then labeled with carboxyfluorescein diacetate succinimidyl ester (0.5 μM) and used in co-culture assays with DCs (T and DC in the ratio of 40:1) or with antibodies.

FACS analysis

For FACS analysis, 5×10^5 cells were incubated in 50 μl phosphate-buffered saline and 1% fetal calf serum with the indicated antibodies (1 μg) for 30 minutes at 4 °C. Cells were then washed twice and analyzed using a FACSCalibur flow cytometer, equipped with CellQuestPro software (BD Biosciences, Heidelberg, Germany).

Mixed lymphocyte reaction and T-cell proliferation

Where indicated, coated anti-CD3 (5 μg ml^{-1}) and soluble anti-CD28 (2.5 μg ml^{-1}) antibodies were used to stimulate T cells. For APC-dependent stimulation (mixed lymphocyte reaction), T cells (1×10^5 per well) and BALB/c-DC (2,500 per well) were used in a ratio of T:DC = 40:1 and as described before (Varga *et al.*, 2007). Where indicated, T cells were additionally cultured with anti-ICAM-1 antibodies (5 μg ml^{-1}). After 5 days of culture, supernatants were taken for analysis of cytokine content, T cells were stained for CD4, and proliferation (carboxyfluorescein diacetate succinimidyl ester dilution) was measured using FACS.

Cytometric bead array

Cytokine production was measured from the supernatants of cell culture and analyzed using cytometric bead array (BD Pharmingen)

according to the manufacturer's instructions. Cytometric bead array FlexSets were used for IFN-γ, IL-2, and IL-4.

Clustering assay

For homotypic aggregation, 2×10^6 syngeneic DC and 10×10^6 $CD18^{+/+}$ or $CD18^{-/-}$ T cells were plated on six-well plates for 24 hours and photographed directly from the culture. For immature DCs, cells of day 7 of culture were used. For mature DCs, day 6 cells were stimulated for 48 hours with 100 ng ml^{-1} lipopolysaccharide.

3D collagen gel

DC–T interactions within 3D collagen gels were analyzed as described before (Gunzer *et al.*, 2000) using an Olympus BX61 microscope (Olympus, Hamburg, Germany) equipped with ''AnalySIS'' software (Olympus, Münster, Germany). In short, 4×10^4 DCs were incubated together with 4×10^5 $CD18^{+/+}$ or $CD18^{-/-}$ T cells in 3D collagen gels in a self-constructed chamber at 37 °C and microscopically recorded with a time-lapse video recorder.

Apoptosis

Apoptosis was measured by staining with annexin V-FITC (STS, Alexis, San Diego, CA) and propidium iodide (1 μg ml^{-1}) as described previously. FACS analysis was performed using CellQuestPro Software (BD Biosciences).

Statistical analysis

Results are mean values ± SEM. $P > 0.05$ were considered not to be significant. Statistical analysis was performed using the Student's *t*-test (two tailed and unpaired; $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Abraham C, Griffith J, Miller J (1999) The dependence for leukocyte function-associated antigen-1/ICAM-1 interactions in T cell activation cannot be overcome by expression of high density TCR ligand. *J Immunol* 162:4399–405

Acuto O, Mise-Omata S, Mangino G *et al.* (2003) Molecular modifiers of T-cell antigen receptor triggering threshold: the mechanism of CD28 costimulatory receptor. *Immunol Rev* 192:21–31

Anderson DC, Miller LJ, Schmalstieg FC *et al.* (1986) Contributions of the Mac-1 glycoprotein family to adherence-dependent granulocyte functions: structure-function assessments employing subunit-specific monoclonal antibodies. *J Immunol* 137: 15–27

Arnaout MA (1990) Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood* 75:1037–50

Camacho SA, Heath WR, Carbone FR *et al.* (2001) A key role for ICAM-1 in generating effector cells mediating inflammatory responses. *Nat Immunol* 2:523–9

Carlos TM, Harlan JM (1994) Leukocyte-endothelial adhesion molecules. *Blood* 84:2068–101

Chirathaworn C, Kohlmeier JE, Tibbetts SA *et al.* (2002) Stimulation through intercellular adhesion molecule-1 provides a second signal for T cell activation. *J Immunol* 168:5530–7

Emoto M, Miyamoto M, Emoto Y *et al.* (2003) Highly biased type 1 immune responses in mice deficient in LFA-1 in Listeria monocytogenes infection are caused by elevated IL-12 production by granulocytes. *J Immunol* 171:3970–6

Grabbe S, Varga G, Beissert S *et al.* (2002) Beta2 integrins are required for skin homing of primed T cells but not for priming naive T cells. *J Clin Invest* 109:183–92

Gunzer M, Schafer A, Borgmann S *et al.* (2000) Antigen presentation in extracellular matrix: interactions of T cells with dendritic cells are dynamic, short lived, and sequential. *Immunity* 13:323–32

Gunzer M, Weishaupt C, Planelles L *et al.* (2001) Two-step negative enrichment of CD4+ and CD8+ T cells from murine spleen via nylon wool adherence and an optimized antibody cocktail. *J Immunol Methods* 258:55–63

Hogg N, Henderson R, Leitinger B *et al.* (2002) Mechanisms contributing to the activity of integrins on leukocytes. *Immunol Rev* 186:164–71

Hynes RO (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69:11–25

Inaba K, Inaba M, Romani N *et al.* (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 176:1693–702

Jenks SA, Miller J (2000) Inhibition of IL-4 responses after T cell priming in the context of LFA-1 costimulation is not reversed by restimulation in the presence of CD28 costimulation. *J Immunol* 164:72–8

Kandula S, Abraham C (2004) LFA-1 on CD4+ T cells is required for optimal antigen-dependent activation *in vivo*. *J Immunol* 173:4443–51

Labeur MS, Roters B, Pers B *et al.* (1999) Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J Immunol* 162:168–75

Lee SH, Prince JE, Rais M *et al.* (2003) Differential requirement for CD18 in T-helper effector homing. *Nat Med* 9:1281–6

Linsley PS, Ledbetter JA (1993) The role of the CD28 receptor during T cell responses to antigen. *Annu Rev Immunol* 11:191–212

Luksch CR, Winqvist O, Ozaki ME *et al.* (1999) Intercellular adhesion molecule-1 inhibits interleukin 4 production by naive T cells. *Proc Natl Acad Sci USA* 96:3023–8

Maraskovsky E, Troutt AB, Kelso A (1992) Co-engagement of CD3 with LFA-1 or ICAM-1 adhesion molecules enhances the frequency of activation of single murine CD4+ and CD8+ T cells and induces synthesis of IL-3 and IFN-gamma but not IL-4 or IL-6. *Int Immunol* 4:475–85

Ni HT, Deeths MJ, Li W *et al.* (1999) Signaling pathways activated by leukocyte function-associated Ag-1-dependent costimulation. *J Immunol* 162:5183–9

Perez OD, Mitchell D, Jager GC *et al.* (2003) Leukocyte functional antigen 1 lowers T cell activation thresholds and signaling through cytohesin-1 and Jun-activating binding protein 1. *Nat Immunol* 4:1083–92

Porter JC, Bracke M, Smith A *et al.* (2002) Signaling through integrin LFA-1 leads to filamentous actin polymerization and remodeling, resulting in enhanced T cell adhesion. *J Immunol* 168:6330–5

Ragazzo JL, Ozaki ME, Karlsson L *et al.* (2001) Costimulation via lymphocyte function-associated antigen 1 in the absence of CD28 ligation promotes anergy of naive CD4+ T cells. *Proc Natl Acad Sci USA* 98:241–6

Salomon B, Bluestone JA (1998) LFA-1 interaction with ICAM-1 and ICAM-2 regulates Th2 cytokine production. *J Immunol* 161:5138–42

Scharffetter-Kochanek K, Lu H, Norman K *et al.* (1998) Spontaneous skin ulceration and defective T cell function in CD18 null mice. *J Exp Med* 188:119–31

Schonlau F, Scharffetter-Kochanek K, Grabbe S *et al.* (2000) In experimental leishmaniasis deficiency of CD18 results in parasite dissemination

associated with altered macrophage functions and incomplete Th1 cell response. *Eur J Immunol* 30:2729–40

Semnani RT, Nutman TB, Hochman P *et al.* (1994) Costimulation by purified intercellular adhesion molecule 1 and lymphocyte function-associated antigen 3 induces distinct proliferation, cytokine and cell surface antigen profiles in human ''naive'' and ''memory'' CD4+ T cells. *J Exp Med* 180: 2125–35

Sims TN, Dustin ML (2002) The immunological synapse: integrins take the stage. *Immunol Rev* 186:100–17

Smits HH, de Jong EC, Schuitemaker JH *et al.* (2002) Intercellular adhesion molecule-1/LFA-1 ligation favors human Th1 development. *J Immunol* 168:1710–6

Springer TA (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76:301–14

Springer TA (1995) Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annu Rev Physiol* 57:827–72

Van der Vieren M, Le TH, Wood CL *et al.* (1995) A novel leukointegrin, alpha d beta 2, binds preferentially to ICAM-3. *Immunity* 3:683–90

Varga G, Balkow S, Wild MK *et al.* (2007) Active MAC-1 (CD11b/CD18) on DCs inhibits full T-cell activation. *Blood* 109:661–9

Vazeux R, Hoffman PA, Tomita JK *et al.* (1992) Cloning and characterization of a new intercellular adhesion molecule ICAM-R. *Nature* 360:485–8

Zhang J, Salojin KV, Gao JX *et al.* (1999) p38 mitogen-activated protein kinase mediates signal integration of TCR/CD28 costimulation in primary murine T cells. *J Immunol* 162:3819–29

Zuckerman LA, Pullen L, Miller J (1998) Functional consequences of costimulation by ICAM-1 on IL-2 gene expression and T cell activation. *J Immunol* 160:3259–68